

Rec'd PTO 17 JUN 2005

PCT/AU03/01711



10/530222

Patent Office
Canberra

RECEIVED	
28 JAN 2004	
WIPO	PCT

I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003900296 for a patent by JAMES COOK UNIVERSITY as filed on 23 January 2003.



WITNESS my hand this
Sixteenth day of January 2004

J. Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

BEST AVAILABLE COPY

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: **Organ arrest, protection, preservation and recovery**

The invention is described in the following statement:

ORGAN ARREST, PROTECTION, PRESERVATION AND RECOVERY

The present invention relates to a pharmaceutical composition for arresting, protecting and/or preserving an organ, tissue or cell. The present invention also provides a method for arresting, protecting and preserving organs, in particular the heart during open-heart surgery, cardiovascular diagnosis, therapeutic intervention, organ transplantation (including xenotransplantation) or when the heart is physiologically damaged or weak. The invention also relates to a method of protecting an organ, tissue or cell from damage, in particular after hypoxia, ischaemia or injury.

There are over 20,000 open-heart surgery operations each year in Australia, over 800,000 in the United States and about 1,000,000 in Europe. Of those requiring open-heart surgery, about 1.2% are neonates/infants primarily as a consequence of congenital heart disease. In the last 10 years, the prolonged use of depolarising potassium cardioplegia has been linked to ionic and metabolic imbalances, myocardial stunning, ventricular arrhythmias, ischaemic injury, microvascular injury, tissue oedema, free radical production and functional loss during the reperfusion period. Indeed, of the about two million patients undergoing open-heart surgery worldwide, about 10% have significant left heart dysfunction in the early period of recovery. The major ion imbalances are thought to be a low pH, activation of the Na^+/H^+ exchanger, increased sodium influx, and activation of the Na/Ca^{2+} exchanger (and Ca^{2+} leak from the sarcoplasmic reticulum) leading to a potentially lethal rise in intracellular Ca^{2+} . The accumulation of Ca^{2+} may occur primarily during reperfusion after short-term arrest, but may occur during the cardioplegic period as well during prolonged arrest.

The heart may be arrested for up to 3 hours during open-heart surgery. High potassium cardioplegia (K^+ in excess of 15-20 mM) has been the conceptual basis of myocardial arrest and protection for over 40 years. Currently the majority of solutions used contain high potassium including the widely used St Thomas Hospital Solution No 2 (commercial name Plegisol) which generally contains 110 mM NaCl, 16 mM KCl, 16 mM MgCl_2 , 1.2 mM CaCl_2 and 10 mM NaHCO_3 and has a pH of about 7.8. High potassium solutions usually lead to a membrane

depolarisation from about -80 to -50mV . Notwithstanding hyperkalemic solutions providing acceptable clinical outcomes, recent evidence suggests that progressive potassium induced depolarisation leads to ionic and metabolic imbalances that may be linked to myocardial stunning, ventricular arrhythmias, ischaemic injury, endothelial cell swelling and dysfunction, microvascular damage, cell death and loss of pump function during the reperfusion period. Infant hearts appear to be more prone to damage with cardioplegic arrest from high potassium than adult hearts. The major ion imbalances postulated are linked to an increased sodium influx which in turn activates the $\text{Na}^+/\text{Ca}^{2+}$ exchangers leading to a rise in intracellular Ca^{2+} . Compensatory responses to these ion and metabolic imbalances include the activation of anaerobic metabolism to replenish ATP with a concomitant decrease in phosphocreatine and glycogen and an increase in tissue lactate and fall in tissue pH. Free radical generation and oxidative stress have also been implicated in potassium arrest and partially reversed by the administration of antioxidants. In some cases, high potassium induced ischaemia has been reported to have damaged smooth muscle and endothelial function. Further damage to the heart can occur during and following ischaemia from activation of the inflammatory process including alteration to blood properties affecting blood flow to myocardial tissue.

Organs, such as hearts, which have been previously damaged or injured, or which are otherwise "sick" or physiologically sub-optimal are naturally even more susceptible to such adverse effects.

In an attempt to minimise ischaemic damage during cardioplegic arrest, an increasing number of experimental studies have employed ATP-sensitive potassium channel openers instead of high potassium^{6-8, 12-14}. Cardioprotection using nicorandil, aprikalim or pinacidil is believed to be linked to the opening of the potassium channel which leads to a hyperpolarised state, a shortening of the action potential and decreasing Ca^{2+} influx into the cell. One shortfall however is that the heart takes the same time or longer to recover with no improvement in function compared to high potassium cardioplegic solutions. Another limitation is that pinacidil requires a carrier due to its low solubility in aqueous solutions. The

carrier routinely used is dimethyl sulphoxide (DMSO) or high concentrations of ethanol (ETOH) which are controversial when used in animal or human therapy¹⁴.

Most investigators, including those who advocate using potassium channel openers, believe that as soon as blood flow is halted and the arrest solution administered, ischaemia occurs and progressively increases with time. To reduce the likelihood of damage, the applicant sought a cardioplegic solution that would place the heart in a reversible hypometabolic state analogous to the tissues of a hibernating turtle, a hummingbird in torpor or an aestivating desert frog. When these animals drop their metabolic rate (some by over 90%), their tissues do not become progressively ischaemic but remain in a down-regulated steady state where supply and demand are closely matched. An ideal cardioplegic solution should produce a readily reversible, rapid electrochemical arrest with minimal tissue ischaemia. The heart should accumulate low tissue lactate, utilise little glycogen, show minimal changes in high-energy phosphates, cytosolic redox (NAD/NADH) and the bioenergetic phosphorylation (ATP/ADP Pi) ratio and free energy of ATP. There should be little or no change in cytosolic pH or free magnesium, minimal water shifts between the intracellular and extracellular phases, and no major ultrastructural damage to organelles such as the mitochondria. The ideal cardioplegic solution should produce 100% functional recovery with no ventricular arrhythmia, cytosolic calcium overload, or other pump abnormalities. There is no cardioplegic solution currently available which fulfils all these requirements.

In light of the potential problems with high potassium cardioplegia, alternative strategies to arrest the heart have been sought^{7,12}. One strategy has been to 'clamp' the membrane potential at or close to its natural resting state of around -83 mV using ATP-sensitive K⁺ channel-openers (pinacidil, nicorandil or aprikalim) whereby it is proposed that fewer channels, exchangers or pumps are activated and the heart arrests in diastole in a profoundly hypometabolic state^{6-8,12-14}. Protection is therefore ensured by minimising metabolic perturbations, reducing transmembrane fluxes and reducing the possibility of Na⁺ and Ca²⁺ loading⁶ and preventing depletion of cellular ATP. However, while the theory appears well-founded, the potential use of K⁺ channel-openers in

normokalemic cardioplegia in the clinical setting remains problematic. This is due in part to the inconclusive results obtained in a variety of animal models ¹⁴ and because K⁺ channel-openers are believed to induce arrest by a different mechanism – K⁺ channel-openers hyperpolarise or polarise the cell membrane whereas high potassium cardioplegia depolarises the cell membrane ⁶.

The applicant previously found that the heart can be better protected by using a potassium channel opener or agonist and/or an adenosine receptor agonist (preferably adenosine) and a local anaesthetic (preferably lignocaine) to arrest, protect and preserve the heart (see WO 00/56145), the entire disclosure of which is incorporated herein by reference.

Adenosine's actions are complex as the drug has many broad-spectrum properties¹⁸. Adenosine has been shown to increase coronary blood flow, hyperpolarise the cell membrane and act as a preconditioning agent via the ATP-sensitive potassium channel and adenosine related pathways including adenosine receptors (A1, A2a, A2b and A3), particularly the A1 receptor¹⁸. Adenosine is also known to improve myocardial recovery as an adjunct to high potassium cardioplegia. Furthermore, adenosine can be used as a pretreatment (whether or not it is present in the arresting solution) to reduce lethal injury. In one study, adenosine was shown to rival potassium arrest solutions and more recently in blood cardioplegia, it reduced post-ischaemic dysfunction in ischaemically injured hearts. Adenosine is sometimes added as an adjunct to potassium cardioplegia.

Lignocaine is a local anaesthetic which is believed to block sodium fast channels and has antiarrhythmic properties by reducing the magnitude of inward sodium current⁶. In this specification, the terms "lidocaine" and "lignocaine" are used interchangeably. The accompanying shortening of the action potential is thought to directly reduce calcium entry into the cell via Ca²⁺ selective channels and Na⁺/Ca²⁺ exchange. Recent reports also implicate lignocaine with the scavenging of free radicals such as hydroxyl and singlet oxygen in the heart during reperfusion. Associated with this scavenging function, lignocaine may also inhibit phospholipase activity and minimise membrane degradation during ischaemia. Lignocaine can also depress vascular relaxations by a complex

mechanism including poly(ADP-ribose) synthetase enzyme activity. Lignocaine has also been shown to have a myocardial protective effect and in one study was found to be superior to high potassium solutions. However, these experiments show that lignocaine alone at 0.5, 1.0 and 1.5 mM gave highly variable functional recoveries using the isolated working rat heart. Lignocaine has also been shown to reduce infarct size in the brain and protect against reperfusion injury in the heart. More recently lignocaine has been shown to exhibit a number of pharmacological actions not related to the sodium channel block²³. For example, recent work has shown that local anaesthetics, including lignocaine, inhibit inflammatory responses. They also have beneficial effects in a number of pathological processes dependent on an overly active inflammatory response such as adult respiratory distress syndrome and in ischaemia-reperfusion injury. Intravenous lignocaine has also been shown to be effective in prevention of deep vein thrombosis after elective hip surgery. Lignocaine therefore appears to be effective in both attenuating inflammatory and hypercoagulable states (post-operative thrombosis) in the clinical setting²³.

However, the combination of the potassium channel opener and local anaesthetic result in arrest and better protection of the organ under normal potassium concentration (ie, physiological levels of potassium), thus reducing the risk of potassium induced injury to the organ which prior art high potassium arrest solutions may induce. This cardioplegia solution containing the combination of the potassium channel opener and local anaesthetic was shown by the applicant to generally improve functional recovery from arrest of the organ over existing solutions.

This invention is thus directed to overcome or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In particular, this invention is directed towards a pharmaceutical composition for arresting, protecting and/or preserving an organ, tissue or cell, with improved recovery of an organ, after arrest of the organ. In one aspect, the organ, tissue or cell is previously injured or damaged. The invention is also directed to a method for arresting, protecting, preserving and/or reducing damage

to a damaged or injured organ. The term "organ" is used herein in its broadest sense and refers to any part of the body exercising a specific function including tissues and cells or parts thereof, for example, cell lines or organelle preparations.

According to another aspect of the present invention, there is provided a
5 pharmaceutical composition for arresting and protecting an organ including an effective amount of a primary potassium channel opener or agonist and/or adenosine receptor agonist and a local anaesthetic. Examples of these components are given below. The invention also provides a method for arresting and protecting an organ, including a damaged or injured organ.

10 In another aspect of the invention, there is provided a composition for arresting and protecting an organ upon administration of a single dose of the composition, the composition including a primary potassium channel opener or agonist and/or adenosine receptor agonist and a local anaesthetic. The invention also provides a method for arresting and protecting an organ comprising
15 administering as a single dose an effective amount of that composition.

In another aspect of the invention, there is provided a composition for arresting and protecting an organ by intermittent administration of the composition, the composition including an effective amount of a primary potassium channel opener or agonist and/or adenosine receptor agonist and a local anaesthetic. A
20 suitable administration schedule is a 2 minute induction dose every 20 minutes throughout the arrest period. The actual time periods can be adjusted based on observations by one skilled in the art administering the composition, and the animal/human model selected. The invention also provides a method for intermittently administering a composition for arresting and protecting an organ.

25 The composition can of course also be used in continuous infusion with both normal and injured organs, such as the heart.

In these embodiments of the invention, the organ may be a heart. In particular, the heart may be a healthy heart or an injured, damaged or "sick" heart.

The composition of the invention has been shown to be particularly advantageous over prior art compositions for administration to injured, damaged or sick hearts.

In one preferred embodiment, the composition includes an effective amount of adenosine and lignocaine.

- 5 In preferred embodiments of the invention, the organ is at a temperature range of between 0 and 38°C. For methods directed to arresting and protecting an organ, the temperature is more preferably 20° to 37°C.

- 10 The invention also provides a use of the composition as described above (together with the preferred embodiments described below) in the methods described above. This use of the composition can extend to many therapeutic applications, including without limitation, cardio-vascular diagnosis, use in treatment of heart attack, resuscitation therapy, short-term and long-term storage of organs tissues or cells (including graft vessels), use before, during and/or following open-heart surgery, angioplasty and other therapeutic interventions.

- 15 A composition according to any of these embodiments of the invention preferably has a potassium concentration of no more than typical blood concentration levels (ie, 4 to 6 mM). However, the composition of the invention is effective with higher potassium concentrations (eg, up to 15 mM) but these are not necessary for efficacy of the composition of the invention.

- 20 Similarly, in these embodiments of the invention, the magnesium concentration is similar to that in blood (ie, 0.5 to 1.5 mM) but again higher concentrations up to around 20 mM can be used if desired.

- 25 Similarly, in these embodiments of the composition, the glucose concentration is similar to that in blood (ie, 5 mM) but higher concentrations can be used if desired.

Without being bound by any theory or mode of action, it is believed that this way of arresting, preserving and protecting a heart (whether healthy or injured)

with the composition involves 'polarising' the cell membrane (-83 mV) and arresting the heart in asystole as opposed to 'depolarising' the membrane at -50 mV using known high K⁺ cardioplegic solutions. This places the heart in a more 'natural' state of suspended animation for which energy requirements are dramatically reduced. This is clinically important because of the link between prolonged high K⁺ induced membrane depolarisation and ischaemia-reperfusion injury, increased incidence of lethal arrhythmias and left ventricular or pump dysfunction. Thus the composition of the invention has the advantage of not only arresting the heart, as with high potassium cardioplegia, but to arrest, protect and preserve the heart. In addition to decreasing energy demand during arrest, the composition incorporates potent anti-inflammatory and anti-coagulable actions which attenuate ischaemic-reperfusion injury under both surgical and non-surgical conditions of reperfusion, which cannot be obtained with the current hypothermic-, tepid-, warm- or normothermic-based potassium-cardioplegia concepts.

In one embodiment, the composition comprises adenosine and lignocaine. In particular, the composition may include adenosine and lignocaine in the weight ratio of about 1:2.

The composition can be infused or administered as a bolus intravenous, intracoronary or any other suitable delivery route as pretreatment for protection during a cardiac intervention such as open heart surgery (on-pump and off-pump), angioplasty (balloon and with stents or other vessel devices) and as with clot-busters (anti-clotting drug or agents).

The composition can also be infused or administered as a bolus intravenous, intracoronary or any other suitable delivery route for protection during a cardiac intervention such as open heart surgery (on-pump and off-pump), angioplasty (balloon and with stents or other vessel devices) and as with clot-busters to protect and preserve the cells from injury.

The composition may also be infused or administered as a bolus intravenous, intracoronary or any other suitable delivery route for protection following a cardiac intervention such as open heart surgery (on-pump and off-

pump), angioplasty (balloon and with stents or other vessel devices) and as with clot-busters to protect and preserve the cells from injury.

In this application, without being bound by this mode of action, protection is thought to involve (i) reducing ion imbalances, in particular calcium ion loading in the cells, and (ii) attenuation of the inflammatory response to injury

Infusion of the composition during pretreatment and ischaemia and reperfusion provides continuous protection from ischaemic tissue injury including protection from lethal arrhythmias. The protection from localised injury and inflammation can also be obtained when placing a stent into a vessel such as during angioplasty. The composition is also used within a polymer or special coating for a stent for use in any vessel of the body including coronary arteries, carotid arteries, or leg arteries of the body.

The potassium channel openers or agonists may be selected from the group consisting of: nicorandil, diazoxide, minoxidil, pinicadil, aprikalim, cromokulim, acetylcholine, NS-1619 (1,3-dihydro-1-[2-hydroxy5(trifluoromethyl)phenyl]5-(trifluoromethyl)2-H-benzimidazol-one), amlodipine, Bay K 8644(L-type)(1,4-dihydro-26-dimethyl-5-nitro-4[2(trifluoromethyl)phenyl]-3-pyridine carboxylic acid (methyl ester)), bepridil HCl (L-type), calciseptine (L-type), omega-conotoxin GVIA (N-type), omega-conotoxin MVIIC (Q-type), cyproheptadine HCl, dantrolene sodium (Ca^{2+} release inhibitor), diltiazem HCl (L-type), flodipine, flunarizine HCl ($\text{Ca}^{2+}/\text{Na}^{+}$), fluspirilene (L-type), HA-1077 2HCl(1-(5 isoquinoliny) sulphonyl) homo piperazine.HCl), isradipine, loperamide HCl, manoalide (Ca^{2+} release inhibitor), nicardipine HCl (L-type), nifedipine (L-type), nifedipine HCl (L-type), nimodipine (L-type), nitrendipine (L-type), pmozide (L- and T-type), ruthenium red, ryanodine (SR channels), taicatoxin, verapamil HCl (L-type), methoxy-verapamil HCl (L-type), amlodipine, felodipine, YS-035 HCl (L-type)N[2(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxy N-nethyl benzene ethaneamine HCl) and AV blockers such as verapamil and adenosine. It will be appreciated that this list includes calcium antagonists as potassium channel openers are indirect calcium antagonists.

Adenosine is particularly preferred as the primary potassium channel opener or agonist. Adenosine is capable of opening the potassium channel, hyperpolarising the cell, depressing metabolic function, possibly protecting endothelial cells, enhancing preconditioning of tissue and protecting from ischaemia or damage. Adenosine is also an indirect calcium antagonist, vasodilator, antiarrhythmic, antiadrenergic, free radical scavenger, arresting agent, anti-inflammatory agent (attenuates neutrophil activation), analgesic, metabolic agent and possible nitric oxide donor.

Suitable adenosine receptor agonists may be selected from: N⁶-cyclopentyladenosine (CPA), N-ethylcarboxamido adenosine (NECA), 2-[p-(2-carboxyethyl)phenethyl-amino-5'-N-ethylcarboxamido adenosine (CGS-21680), 2-chloroadenosine, N⁶-[2-(3,5-demethoxyphenyl)-2-(2-methoxyphenyl)ethyladenosine, 2-chloro-N⁶-cyclopentyladenosine (CCPA), N-(4-aminobenzyl)-9-[5-(methylcarbonyl)-beta-D-ribofuranosyl]-adenine (AB-MECA), ([IS-[1a,2b,3b,4a(S*)]]-4-[7-[[2-(3-chloro-2-thienyl)-1-methyl-propyl]amino]-3H-imidazole[4,5-b]pyridyl-3-yl]cyclopentane carboxamide (AMP579), N⁶-(R)-phenylisopropyladenosine (R-PLA), aminophenylethyladenosine (APNEA) and cyclohexyladenosine (CHA).

In a preferred embodiment, the pharmaceutical composition according to the present invention further includes a secondary potassium channel opener or agonist. The secondary potassium channel opener or agonist may provide additional cellular protection.

Preferably the secondary potassium channel opener is a mitochondrial ATP-sensitive potassium channel opener. More preferably, the mitochondrial ATP-sensitive potassium channel opener is diazoxide. Diazoxide is believed to preserve ion and volume regulation, oxidative phosphorylation and mitochondrial membrane integrity (appears concentration dependent). More recently, diazoxide affords cardioprotection by reducing mitochondrial oxidant stress at reoxygenation [Ozcan, 2002 #1253]. There is also some evidence that the protective effects of potassium channel openers are associated with modulation of reactive oxygen species generation in mitochondria.

Local anaesthetic agents are drugs which are used to produce reversible loss of sensation in an area of the body. Many local anaesthetic agents consist of an aromatic ring linked by a carbonyl containing moiety through a carbon chain to a substituted amino group. In general there are 2 classes of local anaesthetics defined by their carbonyl-containing linkage group. The ester agents include cocaine, amethocaine, procaine and chloroprocaine, whereas the amides include prilocaine, mepivacaine, bupivacaine, mexiletine and lignocaine. At high concentrations, many drugs that are used for other purposes possess local anaesthetic properties. These include opioid analgesics, Beta-adrenoceptor antagonists, anticonvulsants (lamotrigine and lifarizine) and antihistamines. The local anaesthetic component of the pharmaceutical composition according to the present invention may be selected from these classes, or derivatives thereof, or from drugs than may be used for other purposes. Preferably, the component possesses local anaesthetic properties also.

Preferably the local anaesthetic is Lignocaine. Lignocaine is preferred as it is capable of acting as a local anaesthetic probably by blocking sodium fast channels, depressing metabolic function, lowering free cytosolic calcium, protecting against enzyme release from cells, possibly protecting endothelial cells and protecting against myofilament damage. Lignocaine is also a free radical scavenger, an antiarrhythmic and has anti-inflammatory and anti-hypercoagulable properties. Recently, lidocaine and other Class 1 antiarrhythmic drugs have been shown to relax the pig coronary artery by blocking voltage-gated Ca^{2+} channel which led to a reduced cytoplasmic Ca^{2+} concentration - the Na^{+} fast channels did not appear to be involved (2002, Naunyn Schmiedebergs Arch Pharmacol Jan 365 (1) 56-66).

As lignocaine acts as a local anaesthetic by blocking sodium fast channels, it will be appreciated that other sodium channel blockers could be used instead of or in combination with the local anaesthetic in the method and composition of the present invention. Examples of suitable sodium channel blockers include venoms such as tetrodotoxin, and the drugs primaquine, QX, HNS-32 (CAS Registry # 186086-10-2), NS-7, kappa-opioid receptor agonist U50 488, crobenetine, pilsicainide, phenytoin, tocainide, mexiletine, RS100642, riluzole, carbamazepine,

flecainide, propafenone, amiodarone, sotalol, imipramine and moricizine, or any of derivatives thereof.

In another embodiment of the present invention there is provided a pharmaceutical composition according to the present invention, further including
5 an effective amount of an antioxidant.

The antioxidant component of the pharmaceutical composition according to the present invention may be selected from one or more of the group consisting of: allopurinol, carnosine, Coenzyme Q 10, n-acetyl-cysteine, superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GP), catalase and the
10 other metalloenzymes, glutathione, U-74006F, vitamin E, Trolox (soluble form of vitamin E), Vitamin C, Beta-Carotene (plant form of vitamin A), selenium, Gamma Linoleic Acid (GLA), alpha-lipoic acid, uric acid (urate), curcumin, bilirubin, proanthocyanidins, epigallocatechin gallate, Lutein, lycopene, bioflavonoids and polyphenols.

15 Preferably, the antioxidant is allopurinol. Allopurinol is a competitive inhibitor of the reactive oxygen species generating enzyme xanthine oxidase. Allopurinol's antioxidative properties may help preserve myocardial and endothelial functions by reducing oxidative stress, mitochondrial damage, apoptosis and cell death.

20 In another embodiment of the present invention there is provided a pharmaceutical composition according to the present invention, further including an effective amount of a sodium hydrogen exchange inhibitor. The sodium hydrogen exchange inhibitor reduces sodium and calcium entering the cell.

The sodium hydrogen exchange inhibitor may be selected from one or more
25 of the group consisting of amiloride, cariporide, eniporide, triamterene and EMD 84021, EMD 94309, EMD 96785 and HOE 642 and T-162559 (inhibitors of the isoform 1 of the Na^+/H^+ exchanger). Preferably, the sodium hydrogen exchange inhibitor is amiloride. Amiloride inhibits the sodium proton exchanger (Na^+/H^+ exchanger, also often abbreviated NHE-1) and reduces calcium entering the cell.

During ischaemia excess cell protons (or hydrogen ions) are exchanged for sodium via the Na^+/H^+ exchanger.

Aspects of the invention will now be described in more detail as non-limiting examples to better illustrate preferred embodiments of the invention. In the
5 figures:

Figure 1 is a graph comparing a composition according to the invention ("AL") with a prior art composition ("St Thomas" or "ST") by measuring Cardiac Output (as a percentage of pre-arrest output) at various times after arrest of injured rat hearts.

10 Figure 2 is the same as Figure 1 but measuring Aortic Flow recovery.

Figure 3 is the same as Figure 1 but measuring Coronary Flow.

Figure 4 is the same as Figure 1 but measuring Systolic Pressure.

Figure 5 shows data for (a) Coronary Vascular Resistance (CVR) and (b) O_2 consumption measured during cardioplegia delivery at different times during 2
15 and 4 hr arrest of a healthy heart.

Figure 6 is a representative profile of a heart's surface temperature during arrests.

Figure 7 contains Table 1, being the estimates of the membrane potential in the isolated rat heart before and during arrest by adenosine and lidocaine
20 cardioplegia, hyperkalemic St. Thomas Hospital solution No. 2 or 16 M KCl at 37°C .

Figure 8 contains Table 2, being the results of functional recovery following 2 hrs arrest with the 2 hr arrest data reflected in Figure 5.

Figure 9 contains Table 3, being the results of functional recovery following
25 4 hrs arrest with the 4 hr arrest data reflected in Figure 5.

The injured rat hearts and results in Figures 1 to 4 were generated as follows. Animals Adult Male Sprague-Dawley rats (~300g, n=12) were obtained from Animal Resources Center (Canningvale, WA) and JCU's breeding colony. Animals were fed *ad libitum* and housed in a 12 hour light/dark cycle. On the day of experiment rats were anaesthetised with an intraperitoneal injection of Nembutal (Sodium Pentobarbitone; mg/kg body wt) and the hearts rapidly excised (details below). At all times animals were treated in accordance with the James Cook University Guidelines for use of 'Animals for Experimental Purposes' (Ethics approval number A557). Adenosine (A9251 >99% purity) and all other chemicals were obtained from Sigma Chemical Co (Castle Hill, NSW). Lidocaine hydrochloride was purchased as a 2% solution (Ilium) from the local Pharmaceutical Supplies (Lyppard, Queensland).

Adenosine and Lidocaine Arrest solution (a composition according to the invention): 200 μ M adenosine plus 500 μ M lidocaine in 10 mM glucose containing Krebs Henseleit buffer (pH 7.7 at 37°C). The AL arrest solution was filtered using 0.2 μ M filters and maintained at 37°C. The arrest solution was not actively bubbled with 95% O₂/5% CO₂ hence the higher pH (the average pO₂ of the solution was 131 mmHg and pCO₂ of 5-10 mmHg).

Krebs-Henseleit Perfusion buffer: Hearts were perfused in the Langendorff and working mode with a modified Krebs Henseleit buffer containing 10 mM glucose; 117 mM NaCl, 5.9 mM KCl, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, 1.12 mM CaCl₂ (free Ca²⁺=1.07 mM), 0.512 mM MgCl₂ (free Mg²⁺= 0.5 mM), pH 7.4 at 37°C. The perfusion buffer was filtered using a one micron (1 μ M) membrane and then bubbled vigorously with 95%O₂/5%CO₂ for a pO₂ above 600 mmHg. The perfusion buffer was not recirculated.

Modified St. Thomas' Hospital Solution No 2: NaCl (110 mM), KCl (16 mM), MgCl₂ (16 mM), CaCl₂ (1.2 mM), NaHCO₃ (25 mM) pH 7.8. The buffer was filtered using 0.2 μ m filters and maintained at 37°C. The solution was not actively bubbled with 95% O₂/5% CO₂ (The average pO₂ of the solution was 125 mmHg and pCO₂ of 5-10 mmHg). The reason for increasing the bicarbonate concentration from 10 mM to physiological levels of 25 mM was to provide greater

buffering capacity²⁵ thus eliminating the difficulty of adjusting the pH of a weakly buffered solution. The experiments showed no significant differences in heart function after 30 min arrest (n=12) or 4 hours arrest (n= 4) between the traditional and modified St. Thomas' Hospital No 2 solution (data not presented). Glucose was not included in St. Thomas solution based on the findings of Hearse and colleagues who showed glucose (with or without insulin) may be deleterious when used as an additive^{6,26}.

Langendorff and Working Rat Heart preparation: Hearts were rapidly removed from anaesthetised rats and immediately placed in ice-cold Krebs-Henseleit buffer. Excess tissue was removed and the heart was connected via the aorta to a standard Langendorff apparatus with a perfusion pressure of 90 cm H₂O (68 mmHg)²⁷. After tying off the pulmonary veins and superior and inferior vena cava to minimize leaks (< 1 ml/min), the pulmonary artery was cannulated. The preparation was then switched to the working mode and the heart was not placed in a thermostated jacket. The preload was preset at 10 cm H₂O (7.6 mmHg) and the afterload 100 cm H₂O (76 mmHg). Hearts were stabilised for 30 minutes before switching back to Langendorff and administering the arrest solution.

Aortic pressure was measured continuously using a pressure transducer (UFI Instruments, Morro Bay, CA) coupled to a MacLab 2e (ADI Instruments). Systolic and diastolic pressures and heart rate were calculated from the pressure trace using the MacLab software. Arterial and venous perfusate pO₂ and pCO₂, pH and ions (Ca²⁺, Cl⁻, and Na⁺) were measured using a Ciba-Corning 865 blood gas machine. Coronary flow and aortic flow were measured in volumetric cylinders. The initial criteria for exclusion of working hearts during the 30 min equilibration period (before ischaemia) was a heart rate less than 200 beats/min, a systolic pressure less than 100 mmHg and coronary flow less than 10 ml/min. No pacing or cardiac massage was employed during the recovery phase in the working mode.

The effect of a composition according to the invention was tested on the isolated working rat heart following 20 min regional ischaemia produced by ligating the left anterior descending (LAD) coronary artery in the working mode at 37°C. Parallel

studies have shown that the infarct size after 30 min ligation of the LAD in the rat heart is 60 to 70% of the area of risk. Heart rate, aortic pressure, coronary flow, aortic flow and oxygen consumption were measured at 2 and 20 min during coronary artery occlusion.

- 5 After 20 min ischaemia, the ligation snare was removed and hearts were reperfused in working mode for 15 min at 37°C. At 15 min, heart rate, aortic pressure, coronary flow, aortic flow and oxygen consumption were measured just before heart arrest.

- 10 The hearts were then switched to Langendorff mode and 50 ml of one of the tested cardioplegia solutions was delivered at 37°C at a constant pressure head of 90 cm H₂O (68 mmHg). The aorta was then cross-clamped and the heart remained quiescent for 40 min. At 40 min, the cross-clamp was removed and a further volume of cardioplegia was delivered for 2 min via the aorta. This mode of cardioplegia delivery is a single 'one shot' delivery as opposed to 'intermittent'
- 15 (often given as an induction dose plus a 2 min delivery every 20 min throughout the arrest period) or 'continuous' delivery which is given throughout the entire arrest period.

- 20 Hearts were then returned to working mode and recovery was monitored for 45 min at 37°C. Protection was assessed by measuring a number of physiological parameters including aortic and coronary flows, heart rate, recovery of systolic and diastolic pressures which were compared to baseline values. All results are expressed as mean \pm standard error of the mean (SEM). Statistics were performed separately for each of the 30 min, 2 hour and 4 hour protocols. Two-way ANOVA with repeated measures were used to compare discrete variables
- 25 (e.g. coronary resistance, aortic flow, systolic and diastolic pressures, oxygen consumption, external work, delivery supply ratio) over multiple time points between the AL and St. Thomas' treatment groups. The alpha level of significance for all experiments was set at $P < 0.05$.

- 30 After 30 min equilibration and baseline readings there was no significant difference between the two groups of hearts (in all Fig 1, 2, 3 & 4). Nor were their

functional differences during acute ischaemic injury or during the 15 min reperfusion period prior to arrest between the two groups (in all Fig 1, 2, 3 & 4). This verifies the uniformity of the acute injury before cardioplegia was administered.

5 The cardiac output of the heart prior to ischaemic injury, during ischaemic injury (2 and 20 min), at 15 min reperfusion (pre-arrest) and during 45 min recovery following 40 min arrest is shown in Fig. 1. Cardiac output fell by 25-30% as a result of the injury and no significant differences were seen between the two groups. However, following cardioplegia, the AL hearts returned higher cardiac
10 output which after 45 min was not significantly different from the prearrest values. This indicates that little or no left ventricular dysfunction as a result of the AL cardioplegia. In contrast, the St. Thomas' hearts showed markedly reduced function with a return of cardiac output of about 30% of pre-arrest values (or 20% of pre-occlusion or control values).

15 Since cardiac output is the sum of the aortic and coronary flow rates, Fig 2 and 3 show that the major factor responsible for the fall in cardiac output in the AL group was a fall in aortic flow, as coronary flow was surprisingly not different from controls. This result suggests that AL provides superior protection against microvascular damage during cardioplegic arrest, and consistent with our prior
20 data showing that AL hearts have little change in vascular resistance during arrest. The data further demonstrates that the injury during ischaemia was probably localised to the left ventricle whose function was compromised because of ligating the left coronary arteries. In contrast, St Thomas' hearts suffered from both microvascular damage (significantly lower coronary flow) and left ventricle
25 myocyte damage (significantly lower aortic flows) compared to AL arrested hearts.

 The cardiac output and flow data are also supported by the pressures generated by the heart (Fig 4). There were no significant differences in systolic pressures in the AL group at any during recovery following arrest but the St. Thomas hearts could only generate 30% of their pre-arrest and pre-injury values.
30 Similar profiles were found for diastolic pressures, heart rate and oxygen consumption and hydraulic work (data not shown).

In summary, the data in Fig 1, 2 and 3 show that AL cardioplegia provides superior protection during 40 min ischaemic arrest compared to modified St. Thomas. Cardioplegia No 2. While there were no significant differences in cardiac output, aortic and coronary flows before and during regional ischemia at 37°C, the
5 AL hearts recovered with statistically higher function ($P < 0.05$). It is noteworthy that each group of hearts had similar function following ischemia indicating that damage was similar (cardiac output was 60 to 70% of pre-injury values). At 15 min into recovery, the AL hearts recovered about 60% and at 30 min there was
10 100% recovery relative to pre-arrest values (Fig 1). St Thomas hearts on the other hand could only generate around 15-20% of pre-arrest cardiac output in recovery. The same differences were seen in systolic pressure from each cardioplegia group (Fig 4).

Accordingly, it can be seen that the AL composition provides superior arrest, protection and preservation in the acutely injured rat hearts compared to
15 modified St. Thomas hospital solution No 2.

The invention also can be used with healthy hearts as is demonstrated in Figure 5. Figure 5 shows data for (a) Coronary Vascular Resistance (CVR) and (b) O₂ consumption during 2 and 4 hr arrest of a healthy heart. CVR was calculated during the 2 min cardioplegia delivery periods. Values are mean \pm
20 SEM and asterisk shows significance between the two cardioplegia from repeated measures ANOVA ($P < 0.05$). All statistical tests for the 2 and 4 hour AL and St Thomas' arrest protocols were performed separately. For clarity, only the 4 hour arrest data is presented for oxygen consumption and arrest time — no significant differences in the first two hours were found between the 2 and 4 hour arrest
25 protocols.

In the following example, the cardioprotective effects of AL cardioplegia on rat ischaemic myocardium are compared to St. Thomas solution at 22 to 37°C as reflected in Figures 5 to 9. Hearts were rapidly removed from anaesthetised rats and immediately placed in ice-cold Krebs-Henseleit buffer. Excess tissue was
30 removed and each heart was connected via the aorta to a standard Langendorff apparatus with a perfusion pressure of 90 cm H₂O (68 mmHg)²⁴. After tying off

the pulmonary veins and superior and inferior vena cava to minimise leaks (<1 ml/min), the pulmonary artery was cannulated. The preparation was then switched to the working mode. The preload was preset at 10 cm H₂O (7.6 mmHg) and the afterload 100 cm H₂O (76 mmHg). Hearts were stabilised for 30 minutes before switching back to Langendorff and administering the arrest solution (see Multidose Cardioplegia delivery below). Heart rate, aortic pressure, coronary flow, aortic flow and oxygen consumption were measured before, during and following arrest.

Animals: Male Sprague-Dawley rats (323 ± 6 g, $n=47$) were obtained from Animal Resources Center (Canningvale, WA) and JCU's breeding colony. Animals were fed *ad libitum* and housed in a 12 hour light/dark cycle. On the day of experiment rats were anaesthetised with an intraperitoneal injection of Nembutal (Sodium Pentobarbitone; 60 mg/kg body wt) and the hearts rapidly excised (details below) ²⁴. At all times animals were treated in accordance with the James Cook University Guidelines for use of 'Animals for Experimental Purposes' (Ethics approval number A557). Adenosine (A9251 >99% purity) and all other chemicals were obtained from Sigma Chemical Co (Castle Hill, NSW). Lidocaine hydrochloride was purchased as a 2% solution (Ilium) from the local Pharmaceutical Supplies (Lyppard, Queensland).

Aortic pressure was measured continuously using a pressure transducer (UFI Instruments, Morro Bay, CA) coupled to a MacLab 2e (ADI Instruments). Systolic and diastolic pressures and heart rate were calculated from the pressure trace using the MacLab software. Arterial and venous perfusate pO₂ and pCO₂, pH and ions (Ca²⁺, Cl⁻, and Na⁺) were measured using a Ciba-Corning 865 blood gas machine. Coronary flow and aortic flow were measured in volumetric cylinders. The initial criteria for exclusion of working hearts during the 30 min equilibration period was a heart rate less than 200 beats/min, a systolic pressure less than 100 mmHg and coronary flow less than 10 ml/min. No pacing or cardiac massage was employed during the recovery phase in the working mode. Heart surface temperature was measured using a Cole-Palmer thermistor-thermometer (8402-20) every 30 sec throughout 2 hours of arrest. The thermistor probe was

tucked under the left auricle, and placement in the left heart chamber showed similar profiles as sub-auricular placement.

Mode of Multidose Cardioplegic delivery and Experimental Protocol: After the initial induction dose (50 ml) via the aorta in the Langendorff mode at 37°C and at constant pressure of 70 mmHg, the aorta was cross-clamped directly using a plastic aortic clip. For the 2 and 4 hour arrest protocols, cardioplegia was replenished every 18 min, with replenishment for 2 min, after which the cross-clamp was reapplied. The heart was not contained in a temperature-controlled jacket. This mode of cardioplegia delivery was repeated every 18 min until the heart was switched to the working mode.

Determination of Tissue Water and Haemodynamic Calculations: Total tissue water (%) was determined by the difference in wet weight and dry weight divided by wet weight and multiplied by 100. Powdered tissue from a number of hearts in control, during different times of arrest and following recovery were dried to a constant weight at 85° C for up to 48 hours as described by Dobson and Cieslar²⁸.

Coronary vascular resistance (CVR) in megadyne sec cm⁻⁵ during 2 min cardioplegia delivery was calculated by dividing delivery pressure by flow (volume/sec) using the equation:

$$CVR = \frac{1333 \times \text{mm Hg}}{(\text{ml/sec})} \times 10^{-6} \quad (1)$$

where 1 mmHg = 1333 dynes cm⁻² and 10⁻⁶ is a conversion factor from dynes to megadynes

Cardiac oxygen consumption, MVO₂ (μmole O₂/min/g dry wt heart), was calculated from Eqn 2.

$$\begin{aligned}
 MVO_2 &= \frac{(p_aO_2 - p_vO_2)}{(Bp - Vp)} \times \frac{\alpha O_2}{22.40} \times \frac{\text{Coronary Flow}}{\text{gm dry wt}} \times 1000 \quad (2) \\
 &= \frac{\text{mmHg}}{\text{mmHg}} \times \frac{\text{ml/ml}}{\text{ml/mmol}} \times \frac{\text{ml/min}}{\text{gm dry wt}} \times 1000
 \end{aligned}$$

where p_aO_2 and p_vO_2 are the partial pressures of oxygen (mmHg) in the arterial and venous perfusion lines. Bp is the barometric pressure (760 mmHg) and Vp is the water vapour pressure at $37^\circ\text{C} = 47.1$ mmHg. The molar volume for oxygen at standard temperature and pressure (STP) was 22.40 ml/millimole. αO_2 is the Bunsen solubility coefficient defined as that volume of oxygen gas dissolved in one ml of solution at a specified temperature reduced to STP (0°C , 760 mmHg) 29. The αO_2 at 37°C for human plasma is 0.024 ml/ml 30. Coronary flow is measured in ml/min and heart weight expressed as g dry wt.

10 External cardiac work or power output (J/min/g dry wt heart)

$$\begin{aligned}
 &= \frac{(\text{aortic} + \text{coronary flow}) (\times 10^{-6})}{\text{Heart dry weight}} \times \frac{\text{average systolic pressure}}{1} \times \frac{101,325}{760} \quad (3) \\
 &= \frac{\text{ml/min} \cdot \text{m}^3/\text{ml}}{\text{gm dry wt}} \times \frac{\text{mmHg}}{\text{mmHg}} \times \frac{\text{Nm}^{-2}}{\text{mmHg}}
 \end{aligned}$$

where 10^6 is required to convert 1 ml into cubic meters and $1 \text{ atm} = 760 \text{ mmHg} = 101,325 \text{ Newton meters}^{-2} (\text{Nm}^{-2})$.

All results are expressed as mean \pm standard error of the mean (SEM).

15 Statistics were performed separately for each of the 30 min, 2 hour and 4 hour protocols. Two-way ANOVA with repeated measures were used to compare discrete variables (e.g. coronary resistance, aortic flow, systolic and diastolic pressures, oxygen consumption, external work, delivery supply ratio) over multiple time points between the AL and St. Thomas' treatment groups. The alpha level of significance for all experiments was set at $P < 0.05$.

During the pre-arrest (or control period) there was no significant difference in functional parameters between the two groups tested: see Table 2 in Figure 8 and Table 3 in Figure 9. Hearts receiving adenosine and lidocaine (AL) cardioplegia achieved electrical and mechanical arrest in 25 ± 2 sec ($n=23$) compared to 70 ± 5 sec ($n=24$) for St. Thomas' hearts. After the 50 ml induction volume, 9 out of 23 AL hearts experienced 1.3 ± 0.2 escape beats followed by total arrest. St. Thomas' hearts arrested by becoming progressively weaker (on the basis of developed aortic pressure) over a longer period of time and generally no escape beats were detected.

Functional data from healthy (non-injured) rat hearts arrested using multidose cardioplegia for 2 and 4 hours are also shown in Tables 2 and 3 respectively. St. Thomas' hearts showed significantly lower functional recoveries than hearts arrested with AL cardioplegia. Mean aortic flow was about 22% and 5-10% of pre-arrest values after 2 and 4 hours arrest respectively. Similarly, systolic pressures were 70 and 30 mmHg for 2 and 4 hours respectively. For the 2 hr St. Thomas' group, heart rate, coronary flow, rate-pressure product and O_2 consumption recovered to 40-50% of their pre-arrest values (Table 2). After 60 min of reperfusion, the 4 hour St. Thomas' group had only 32% of heart rate, 23% of systolic pressure, 5% of aortic flow, 16% of coronary flow and 14% of rate-pressure product (Table 3). In direct contrast, the AL Hearts after 2 and 4 hours arrest recovered up to 77% and 70% of their pre-arrest aortic flows respectively, and systolic pressures also reached 113 to 118 mmHg which were 85 to 100% of pre-arrest values, as were oxygen consumption and rate-pressure product (Tables 2 & 3).

Total tissue water content in the pre-arrest working mode was $86.6 \pm 1.1\%$ ($n=4$) and in agreement with earlier studies of Masuda, Dobson and Veech²⁴. Total tissue water content measured on separate hearts at different times during arrest for St. Thomas' and AL hearts was $87 \pm 0.8\%$ ($n=8$) and $88.7 \pm 0.3\%$ ($n=14$) respectively ($P<0.05$). There were no significant differences found *within* each cardioplegia group (ie AL and St Thomas) after 30 min, 2 hr or 4 hr. Separate measurements on different hearts were also made at reperfusion and recovery. The average values during 60 min reperfusion were $86.5 \pm 0.6\%$ ($n=14$)

and $89.2 \pm 0.3\%$ ($n=20$) for St. Thomas' and AL hearts respectively. As in arrest, AL hearts had significantly higher post-reperfusion water content than St. Thomas' hearts ($P<0.05$), but the increased water content had little adverse effect on functional recovery.

5 In summary, only 50% of St. Thomas hearts (4 out of 8) arrested using
 multidose cardioplegia for 2 hrs could develop aortic flow against an afterload of
 100 cm H₂O, and that percentage dropped to 17% (1 out of 7) in the 4 hour arrest
 group (Fig. 5). In contrast, 100% of hearts arrested with AL cardioplegia
 recovered aortic flow against 100 cm H₂O after 2 hours ($n=7$) and 4 hours ($n=9$)
 10 (Tables 2 and 3).

A representative profile of the heart surface temperature for either AL
 hearts or St. Thomas' hearts is shown in Fig 6. During the control and 1 hour
 reperfusion periods, heart temperature was 37°C but during arrest it cycled
 between 35 and 22°C. The cycling occurred because the heart was not placed in
 15 a temperature-controlled jacket and the peak temperatures correspond to the 2
 min delivery of cardioplegia at 37°C and the valley's to the end of the 18 min 'on-
 clamp' period. The average heart temperature over 2 hours of arrest was 28-30°C
 and was not different between AL and St. Thomas' hearts (Fig 6).

Cardioplegia Delivery Volumes, Coronary Vascular Resistance, and O₂
 20 Consumption during 2 min Off-Clamp: The total cardioplegia volume delivered
 over 4 hours to AL hearts was 273 ml and 201 ml for St. Thomas' hearts, with the
 greatest difference between 2 and 4 hours of arrest. For example, at 240 min, 17
 ml of cardioplegia was delivered to AL hearts and 7.3 ml to St. Thomas' hearts.
 Coronary vascular resistance (CVR) at different cardioplegia delivery times during
 25 2 and 4 hour arrest is shown in Fig 5a. After 2 hours, AL hearts had significantly
 lower resistance than St. Thomas' Hearts ($P<0.05$) which helps explain the higher
 cardioplegia volumes. Decreased CVR is in accord with adenosine's potent
 coronary vasodilatory properties¹⁸.

Oxygen consumption was significantly higher during infusions of
 30 cardioplegia in AL hearts than the St. Thomas' hearts (Fig 5b). The higher O₂

consumption (1.5 to 3 times) was due to both an increase in perfusate inflow-outflow (A-V) pO_2 difference (the average A-V pO_2 difference over 4 hours was 83 ± 1.6 mmHg for AL hearts, and 62 ± 1.9 mmHg for St. Thomas' hearts) and higher flows in AL hearts (lower resistance). During infusions of cardioplegia, oxygen
 5 consumption in AL and St Thomas' hearts fell to 10% and 5% of their pre-arrest controls respectively.

This example shows that the arresting combination of 200 μ M adenosine and 500 μ M lidocaine (AL) in normokalemic Krebs-Henseleit at pH 7.4 and 37°C is superior to hyperkalemic St. Thomas' Hospital solution during prolonged arrest.
 10 Rat hearts arrested with multidose AL cardioplegia showed significantly faster electromechanical arrest times (25 vs 70 sec, $P < 0.05$), had lower coronary vascular resistance during cardioplegia infusions (Fig. 5) and superior functional recoveries following arrest.

Without being bound by any theory or mode of action, it is believed that
 15 possible reasons for AL's superiority over modified St. Thomas' hospital solution may include: Faster arrest times in AL hearts may lead to better preservation of high-energy phosphates and glycogen, and the maintenance of a high cytosolic phosphorylation ($[ATP]/[ADP]$ $[P_i]$) ratio and $\Delta G'_{ATP}$ and low redox (lactate/pyruvate) ratios.

20 Second, superior protection may be linked to adenosine's ability to open sarcolemmal ATP-sensitive K^+ channels of conduction cells and myocytes, shorten the action potential duration, arrest the heart ^{16,17} and protect the myocardium during ischaemia ^{7,9}. Adenosine's negative chronotropic and dromotropic effects are believed mediated in part by activation of A1 receptors and
 25 opening of sarcolemmal ATP-sensitive K^+ channels (via reduction of adenylyl cyclase activity) ¹⁸. This leads to direct and indirect slowing of the heart by inhibiting the pacemaking current in the SA node and slowing atrioventricular (AV) nodal electrical conduction. The A1 receptors are also implicated in the nucleoside's ability to blunt the stimulatory effects of catecholamines, and
 30 inhibition of norepinephrine release from nerve terminals ¹⁸. In addition to adenosine's arresting properties, there is substantial experimental evidence for its

cardioprotective effects during ischemia such as reductions in infarct size, reduced myocardial stunning, free radical scavenging, anti-inflammatory properties (see below) and improved maintenance of cell metabolism ^{18,22}. Activation of ATP-sensitive potassium channels by adenosine is believed to reduce sodium and calcium loading by myocardial cells, and thereby reduce the extent of necrosis, myocardial stunning and reperfusion injury ^{15,21,31}. A role for an adenosine-linked opening of mitochondrial ATP-sensitive channels in negative chronotropy and cardioprotection remains to be clarified.

A third reason for AL cardioplegia's superiority is associated with lidocaine's pharmacological action to close Na^+ fast channels leading to anaesthesia and augmentation of adenosine's arresting effects ⁶. Lidocaine will 'clamp' the membrane potential near or at its resting state and, since fewer channels or pumps are activated at polarised potentials, its actions may have energy sparing effects and further reduce Na^+ and Ca^{2+} loading (see above) ^{9,19,21}. The possibility also exists that lidocaine in combination with adenosine may exert additional arresting and cardioprotective actions through some unknown membrane receptor-ligand and/or channel mediation mechanism(s).

A fourth factor contributing to the superior arrest, protection and preservation of AL cardioplegia is adenosine's potent coronary vasodilatory properties leading to reduced coronary vascular resistance and greater delivery of cardioplegia. The lower coronary resistance in AL hearts was not due to reduced tissue oedema (88.7%), nor was St. Thomas's higher resistance and poor performance due to increased oedema (87%). It is particularly noteworthy that total tissue water in crystalloid perfused rat hearts range from 85 to 88% ²⁴, and significantly higher than *in situ* rat hearts (79%) ²⁸. Crystalloid perfused hearts undergo a major redistribution of tissue water with the extracellular space over two times the *in situ* value ^{24,28}. Further studies are required to investigate the effect of AL cardioplegia on the regulation of coronary blood flow over prolonged arrest periods and the distribution of water in the interstitial, extracellular and intracellular compartments.

A fifth important factor for AL's superiority is adenosine's ¹⁸ and lidocaine's ²³ anti-inflammatory effects which may inhibit cytokine and complement generation that would have a direct effect on myocytes in crystalloid perfused system ¹⁸. The use of adenosine in cell-free systems has been shown to be
5 cardioprotective independent of its effects on neutrophils and other blood-borne inflammatory components ¹⁸. However, adenosine's and lidocaine's anti-inflammatory effects is expected to be of greater importance in blood cardioplegia in intact animal models undergoing cardiopulmonary bypass.

Lastly, AL's superiority over modified St. Thomas' solution may be
10 associated with other compositional differences. AL cardioplegia contains non-depolarising 'physiological' potassium concentration similar to the concentration found in blood. High 'depolarising' potassium cardioplegia has been linked to metabolic imbalances and dearrangements in sarcolemma ion gradients (particularly Ca^{2+}) and left ventricular dysfunction, which is more pronounced at
15 higher arrest temperatures ^{7,9-11,32}. In 1991 Yacoub and colleagues also reported that high potassium in St. Thomas' solution or Bretschneider solution resulted in endothelial damage and concentration dependent ³³. AL cardioplegia also has a lower more 'physiological' magnesium concentration (~0.5 mM), and while 16 mM in St. Thomas' solution has been shown to be cardioprotective ⁶, the lower
20 concentration did not appear to compromise AL heart's performance. Notwithstanding the complexity of these compositional differences, superior protection and preservation of AL cardioplegia may be due to the presence of exogenous glucose (10 mM). As discussed earlier, glucose was omitted from the St. Thomas solution because Hearse and colleagues showed that its presence
25 was detrimental to recovery ^{6,26}, and because commercially available Plegisol (Abbott) does not contain glucose.

The ideal temperature for cardioplegia remains controversial. During open-heart surgery, the surface temperature of the heart under normothermic arrest can drift from 37°C to 32°C. In an attempt to approximate this in the isolated rat heart
30 model, cardioplegia was delivered at 37°C for 2 min every 18 min and the heart temperature permitted to drift during the 'on' clamp period (intermittent ischaemic

period). The heart surface temperature between infusions was 37°C to 22-24°C. Although hearts receiving both AL and modified St Thomas' cardioplegia experienced the same moderate temperature falls during arrest, the protocol used in the examples is different from current normothermic surgical arrest practices. In this study, shifting from lower arrest temperatures to normothermia at reperfusion may have influenced the recovery of St Thomas hearts. A degree of hyperkalemic-induced heart block cannot be ruled out, but this is considered unlikely as there was no sign of electrical disturbance in the St Thomas' group after 30 min arrest (time to first beat was 2 min 13 sec for St. Thomas' hearts and 2 min 27 sec for AL hearts, and both groups developed aortic flow ~5 min). In the working heart model, unlike the intact animal, the perfusion pressure is independent of the development of forward flow (or stroke volume), hence, perfusion pressure during the early moments of reperfusion, when contractile effort was unstable and inconsistent, was similar between both groups. Reasons for poor performance in St Thomas' hearts is more likely related to the precipitous rise in coronary vascular resistance *during* 2 and 4 hours of arrest (up to 4 fold higher than AL hearts) and ischaemia-reperfusion injury. Furthermore, the lower myocardial temperatures achieved between infusions of cardioplegia may have adversely effected the actions of adenosine by blunting the receptor-mediated effects by disengaging the transduction mechanisms ³⁶. However, temperature-related uncoupling of receptor transduction mechanisms may occur at more profound levels of hypothermia. In the present study, AL cardioplegia was associated with greater functional recovery despite the moderate temperature decreases between infusions of cardioplegia.

In a further example, the effect of normokalemic AL cardioplegia on the membrane potential in the heart is described. This example shows the effect of AL cardioplegia on the membrane potential of healthy (non-injured, non-ischaemic) rat hearts, compared with St Thomas Hospital solution No 2, and 16 mM KCl.

Animals Adult Male Sprague-Dawley rats (~300g, n=18) were obtained from Animal Resources Center (Canningvale, WA) and JCU's breeding colony. Animals were fed ad libitum and housed in a 12 hour light/dark cycle. On the day

of experiment rats were anaesthetised with an intraperitoneal injection of Nembutal (Sodium Pentobarbitone; mg/kg body wt) and the hearts rapidly excised (details below). At all times animals were treated in accordance with the James Cook University Guidelines for use of 'Animals for Experimental Purposes' (Ethics approval number A557). Adenosine (A9251 >99% purity) and all other chemicals were obtained from Sigma Chemical Co (Castle Hill, NSW). Lidocaine hydrochloride was purchased as a 2% solution (Ilium) from the local Pharmaceutical Supplies (Lyppard, Queensland).

Estimation of the Myocardial Cell Membrane Potential: Control (non-injured, non-ischaemic, pre-arrest) hearts were freeze-clamped at liquid nitrogen temperatures in the working mode (n=6). A separate group (n=6) was perfused in the working mode and then switched to the Langendorff mode and arrested using St. Thomas' hospital solution No 2 at 37°C. A third separate group (n=6) was perfused in the working mode and then switched to the Langendorff mode and arrested using AL cardioplegia. A few minutes after the hearts were arrested, the hearts were freeze-clamped at liquid nitrogen temperatures and the left ventricular tissue was ground at liquid nitrogen temperatures in a pre-cooled mortar. The tissue was then transferred to liquid nitrogen cooled tubes and kept at -80°C until use.

Tissue (100 mg) was acid-digested for total potassium measurement and left overnight using the methods described in Masuda, Dobson and Veech²⁴. The total tissue potassium concentration and intracellular concentration ([K⁺]_{in}) was measured and calculated using the methods described in Masuda, Dobson and Veech²⁴. The membrane potential was calculated from the Nernst equation, where E_m (membrane potential) = $E_k = \frac{RT}{ZF} \log \left(\frac{[K^+]_{out}}{[K^+]_{in}} \right)$, R is the universal gas constant, T is the temperature in Kelvin, Z is the valence of the ion (1+ for potassium) and F is the Faradays constant. The extracellular potassium ([K⁺]_{out}) is assumed to be the same as in the Krebs-Henseleit (5.9 mM) or cardioplegia (St Thomas, 16 mM; and AL arrest solution, 5.9 mM),

The results show that using the Nernstian distribution of potassium across the heart cell membrane the membrane potential for St Thomas Hospital solution

No 2 was -48 ± 3 mV ($n=6$) (Table 1 in Figure 7). This result is consistent with the accepted published values based on direct potassium electrode measurements. The published values for hyperkalemic 16 mM K⁺ solutions such as St Thomas Hospital solution No 2 or potassium chloride (KCl) are - 50 and 49.5 mV respectively (Table 1). Using the Nernstian method, the membrane potential calculated for the non-injured, non-ischaemic, pre-arrested rat heart was -83 mV, which again is consistent with published values for the isolated perfused rat heart or guinea pig heart. Using the Nernstian distribution of potassium, the membrane potential calculated for isolated rat hearts arrested using AL cardioplegia, was -83 mV. The membrane potential for AL arrested hearts is not different from the resting membrane potential. The results also add further support that the Nernst equation and electrodes agree as a measure of the voltage (potential) difference across the myocardial membrane in the control and arrested state.

Thus, it can be seen that one embodiment of the present invention utilising the arresting combination of a K⁺ channel opener and local anaesthetic (for example, adenosine and lidocaine cardioplegia) does not depolarise the heart cell as high potassium solutions such as St Thomas Hospital solution No 2 or 16 mM KCl (-49.5 to -50 mV), but *polarises* or 'clamps' it close to the resting membrane potential (-83 mV).

Throughout this specification, unless stated otherwise, where a document, act or item of knowledge is referred to or discussed, this reference or discussion is not an admission that the document, act or item of knowledge, or any combination thereof, at the priority date, was part of the common general knowledge.

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

References

1. Ringer S. A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *J. Physiol.* 1883;4:29-42.

2. Hooker DR. On the recovery of the heart in electric shock. *Amer. J. Physiol.* 1929;91:305-328.
3. Melrose DG, Dreyer, B., Bentall, H.H., and Baker, J. B. E. Elective cardiac arrest. *Lancet.* 1955;2:21-22.
- 5 4. Bretschneider HJ, Huubner, G. and Knoll, D. Myocardial resistance and tolerance to ischemia: physiological and biochemical basis. *J. Cardiovas. Surg.* 1975;16:241-260.
5. Buckberg GD. Update of Current Techniques of myocardial protection. *Ann. Thorac. Surg.* 1995;60:805-814.
- 10 6. Chambers DJ, and Hearse, D. J. Cardioplegia and Surgical Ischaemia. In: Sperelakis N, Kurachi, Y., Terzic, A., and Cohen, M.V., ed. *Heart Physiology and Pathophysiology.* San Diego: Academic Press; 2001:887-926.
7. Cohen NM, Damiano, R.J., and Wechsler, A. S. Is there an alternative to potassium arrest? *Ann. Thorac. Surg.* 1995;60:858-863.
- 15 8. Jayawant AM, Stephenson, E.R., Matte, G. S., Prophet, G. A., LaNoue, K.F., Griffiths, J. W., and Damiano, R. J. Potassium channel opener cardioplegia is superior to St. Thomas' solution in the intact animal. *Ann. Thorac. Surg.* 1999;68:67-74.
9. Lopez JR, Jahangir, R., Jahangir, A., Shen, W. K., and Terzic, A.
- 20 Potassium channel openers prevent potassium-induced calcium loading of cardiac cells: possible implications in cardioplegia. *J. Thorac. Cardiovasc. Surg.* 1996;112:820-831.
10. Jayawant AM, and Damiano, R. J. The superiority of pinacidil over adenosine cardioplegia in blood perfused isolated hearts. *Ann. Thorac. Surg.* 25 1998;66.

11. Suleiman MS, Halestrap, A. P., and Griffiths, E. J. Mitochondria: a target for myocardial protection. *Pharmacol. Ther.* 2001;89:29-46.
12. Menasche P, Kevelaitis, C., Grousset, C., Piwnica, A., and Bloch, G. Preconditioning with potassium channel openers. A new concept for enhancing
5 cardioplegic protection. *J. Thorac. Cardiovasc. Surg.* 1995;110:1606-1613.
13. Gross GJ, and Auchampach, J.A. Role of ATP dependent potassium channels in myocardial ischaemia. *Cardiovasc. Res.* 1992;26:1011-1016.
14. Ducko CT, Stephenson, E. R., Jayawant, A. M., Vigilance, D. W., and Damiano, R. J. Potassium channel openers: are they effective as pretreatment or
10 additives to cardioplegia? *Ann. Thorac. Surg.* 2000;69:1363-1368.
15. Hu K, Li, G-R., and Nattel, S. Adenosine-induced activation of ATP-sensitive K^+ channels in excised membrane patches is mediated by PKC. *Amer. J. Physiol.* 1999;276:H488-H495.
16. Foker JE, Einziz, S., and Wang, T. Adenosine metabolism and myocardial
15 preservation. *J. Thorac. Cardiovasc. Surg.* 1980;80:506-516.
17. Schubert R, Vetter, H., Owen, P., Reichart, B., and Opie, L. H. Adenosine Cardioplegia. *J. Thorac. Cardiovasc. Surg.* 1989;98:1057-1065.
18. Vinten-Johansen J, Thourani, V. H., Ronson, R. S. Jordan, J.E., Zhao, Z. Q., Nakamura, M., Velez, D., and Guyton, R. A. Broad-spectrum cardioprotection
20 with adenosine. *Ann. Thorac. Surg.* 1999;68:1942-1948.
19. Haworth RA, Goknur, A. B., Berkoff, H. A. Inhibition of Na-Ca exchange by general anaesthetics. *Circ. Res.* 1989;65:1021-1028.
20. Hearse DJ, O'Brian, K., and Braimbridge, M.V. Protection of the myocardium during ischemic arrest: dose response curves for procaine and
25 lignocaine solutions. *J. Thorac. Cardiovasc. Surg.* 1981;81:873-879.

21. Jovanovic A, Lopez, J. R., Alekseev, A. E., Shen, W. K., and Terzic, A. Adenosine prevents K⁺-induced Ca²⁺ loading: Insight into cardioprotection during cardioplegia. *Ann. Thorac. Surg.* 1998;65:586-591.
22. Lasley RD, and Mentzer, R. M. Protective effects of adenosine in the
5 reversibly injured heart. *Ann of Thorac Surg.* 1995;60:843-846.
23. Hollmann MW, Difazio, C.A., and Durieux, M.E. Ca-signaling G-protein-coupled receptors: a new site of local anesthetic action? *Reg Anesth Pain Med.* 2001;26:565-571.
24. Masuda T, Dobson, G.P., and Veech, R.L. The Gibbs-Donnan near-
10 equilibrium system of heart. *J. Biol. Chem.* 1990;265:20321-20334.
25. Dobson GP, Veech, R.L., Hoeger, U., and Passonneau, J.V. Enzymatic determination of total CO₂ in freeze-clamped animal tissues and plasma. *Anal. Biochem.* 1991;195:232-237.
26. Hearse DJ, Stewart, D.A., Braimbridge, M.V. Myocardial protection during
15 ischemic cardiac arrest. Possible deleterious effects of glucose and mannitol in coronary infusates. *J. Thorac Cardiovasc Surg.* 1978;76:16-23.
27. Neely JR, and Rovetto, M. J., ed. *Techniques for perfusing isolated rat hearts.* London. New York: Academic Press; 1975.
28. Dobson GP, and Cieslar, J.H. Intracellular, interstitial and plasma spaces in
20 the rat myocardium in vivo. *J. Mol. Cell Cardiol.* 1997;29:3357-3363.
29. Altman PL, and Dittmer, D. S., ed. *Respiration and Circulation.* Bethesda, Maryland: Federation of American Societies for Experimental Biology; 1971.
30. Christoforides C, Laasberg, L. H., and Hedley-Whyte, J. Effect of temperature on solubility of O₂ in human plasma. *J. Appl. Physiol.* 1969;26:56-60.

31. Grover GJ, and Garlid, K.D. ATP-Sensitive potassium channels: a review of their cardioprotective pharmacology. *J Mol Cell Cardiol.* 2000;32:677-695.
32. Ou R, Gavin, J. B., Esmore, D. S., and Rosenfeldt, F. L. Increased temperature reduces the protective effect of university of Wisconsin solution in the heart. *Ann. Thorac. Surg.* 1999;68:1628-1634.
33. Mankad PS, Chester, A. H., and Yacoub, M. H. Role of potassium concentration in cardioplegic solutions in mediating endothelial damage. *Ann. Thorac. Surg.* 1991;51:89-93.
34. Cohen NM, Wise, R. M., Wechsler, A. S., and Damiano, R.J. Elective cardiac arrest with a hyperpolarising adenosine triphosphate-sensitive potassium channel opener: A novel form of myocardial protection? *J. Thorac. Cardiovasc. Surg.* 1993;106:317-328.
35. Jayawant AM, Lawton, J.S., Hsia, P.W., and Damiano, R. J. Hyperpolarised cardioplegic arrest with Nicorandil. *Circulation (9 Suppl. 2).* 1997;96:11 240-246.
36. Lorenzen A, Guerra, L., Vogt, H., and Schwabe, U. Interaction of full and partial agonists of the A1 adenosine receptor with receptor/G protein complexes in rat brain membranes. *Mol Pharmacol.* 1996;49:915-926.

Figure 1

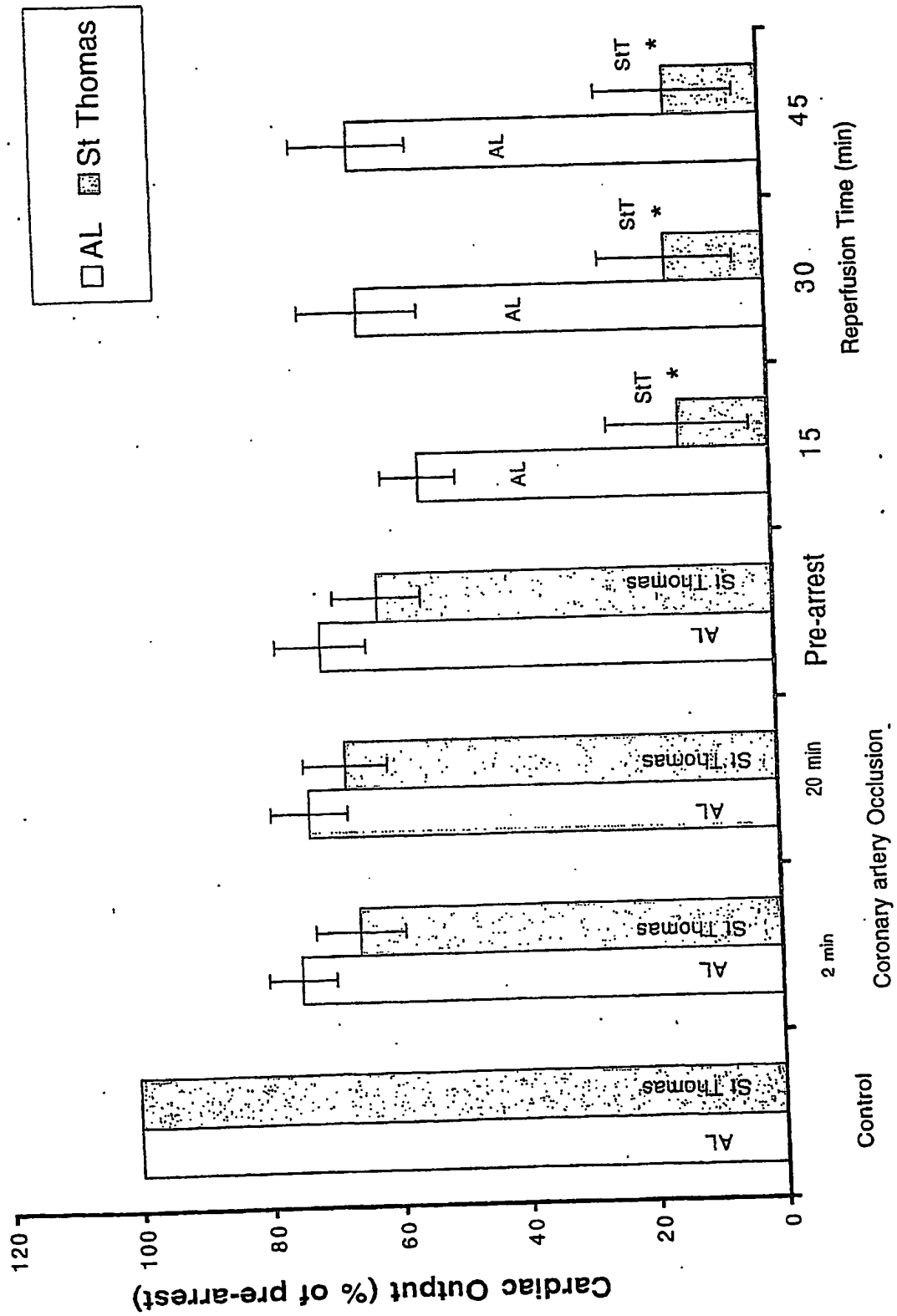


Figure 2

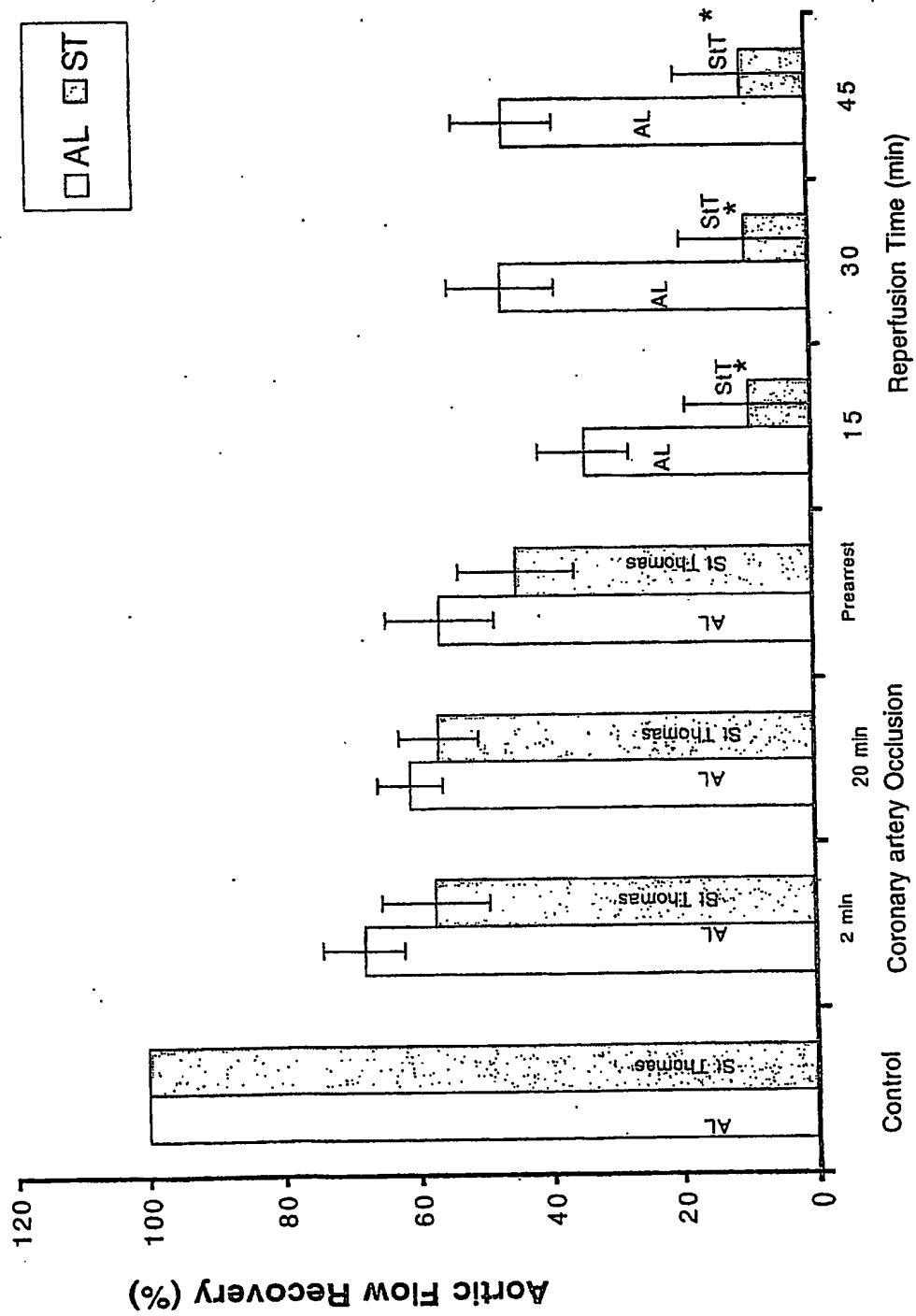


Figure 3

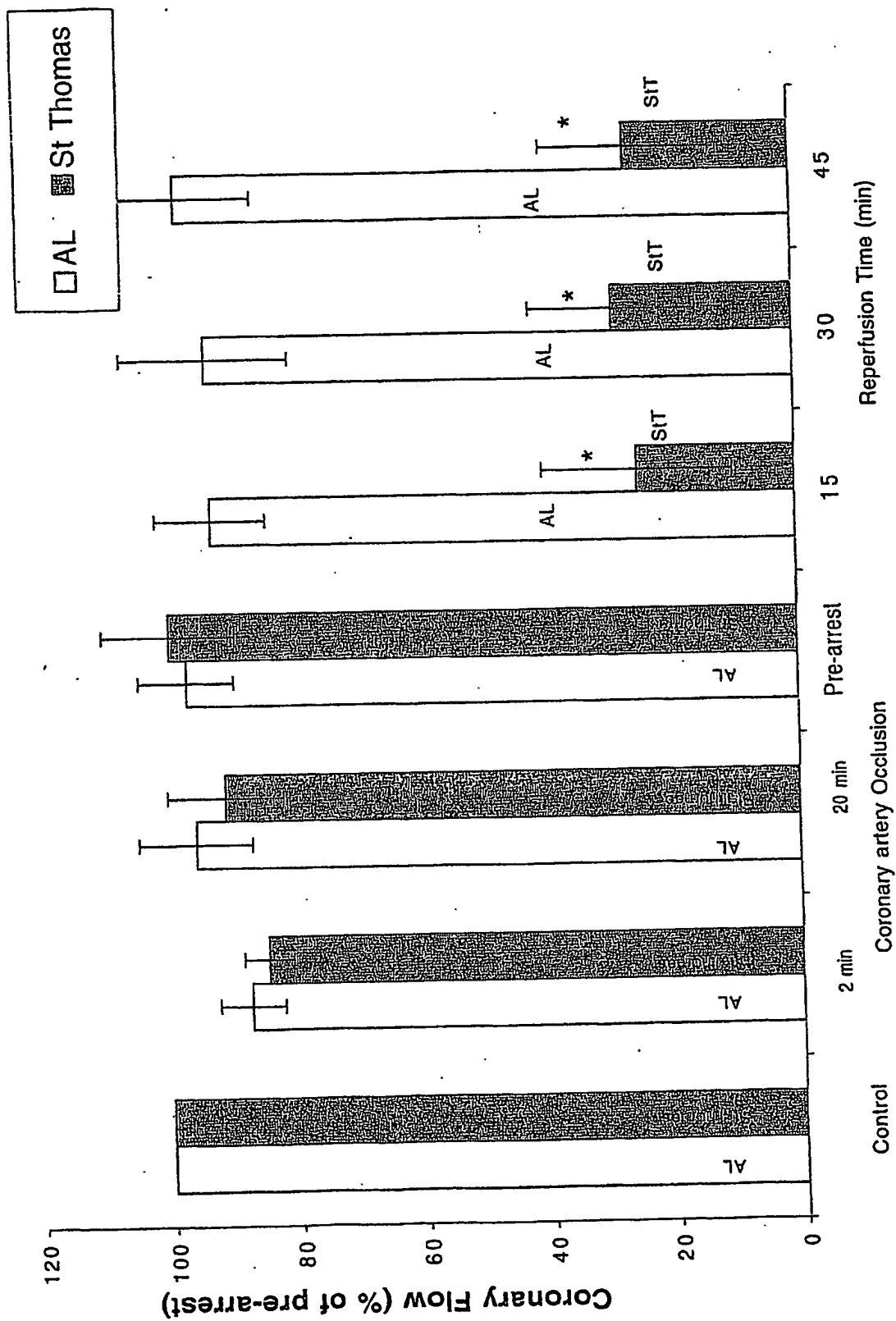


Figure 4

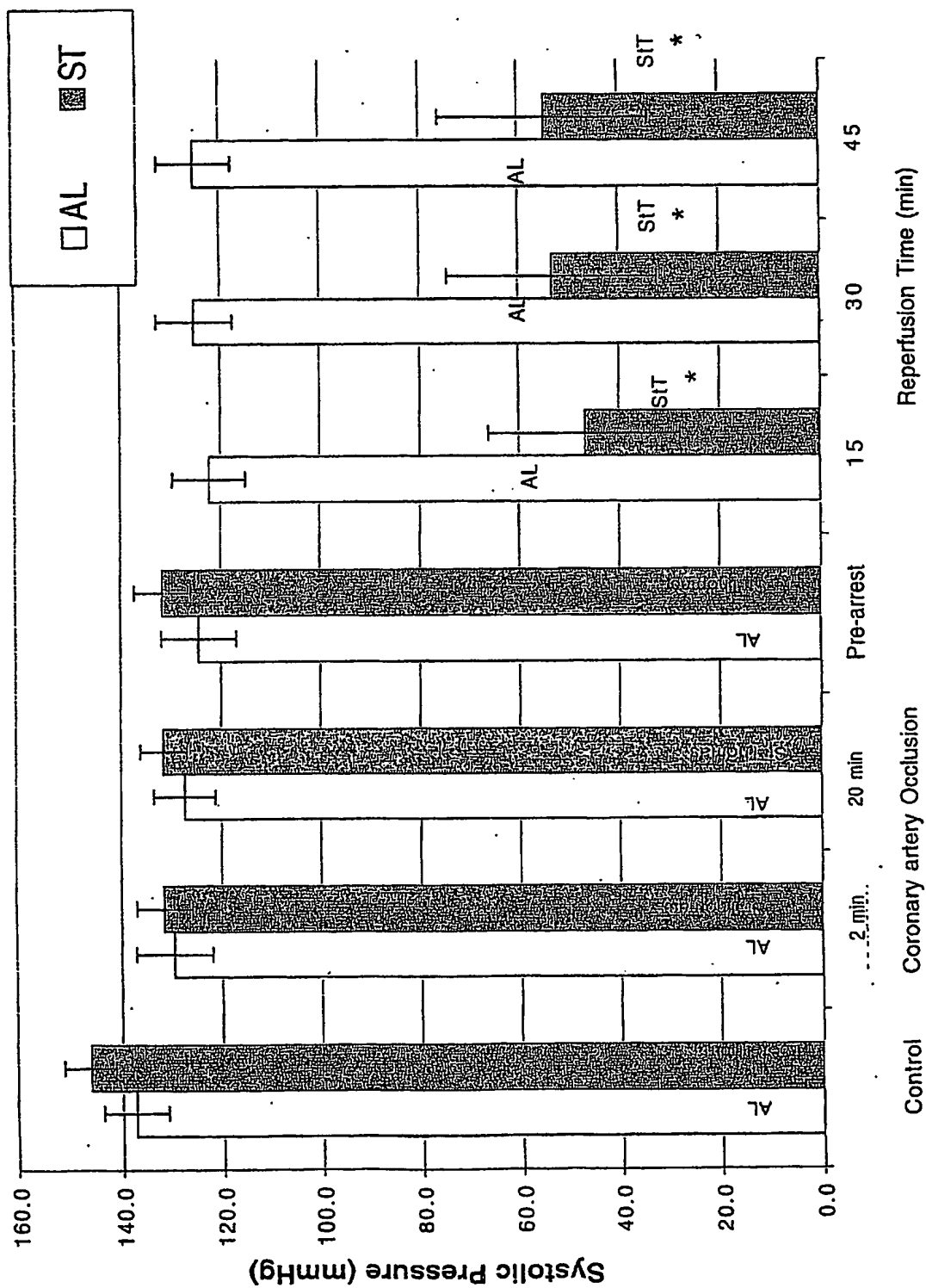


Figure 5

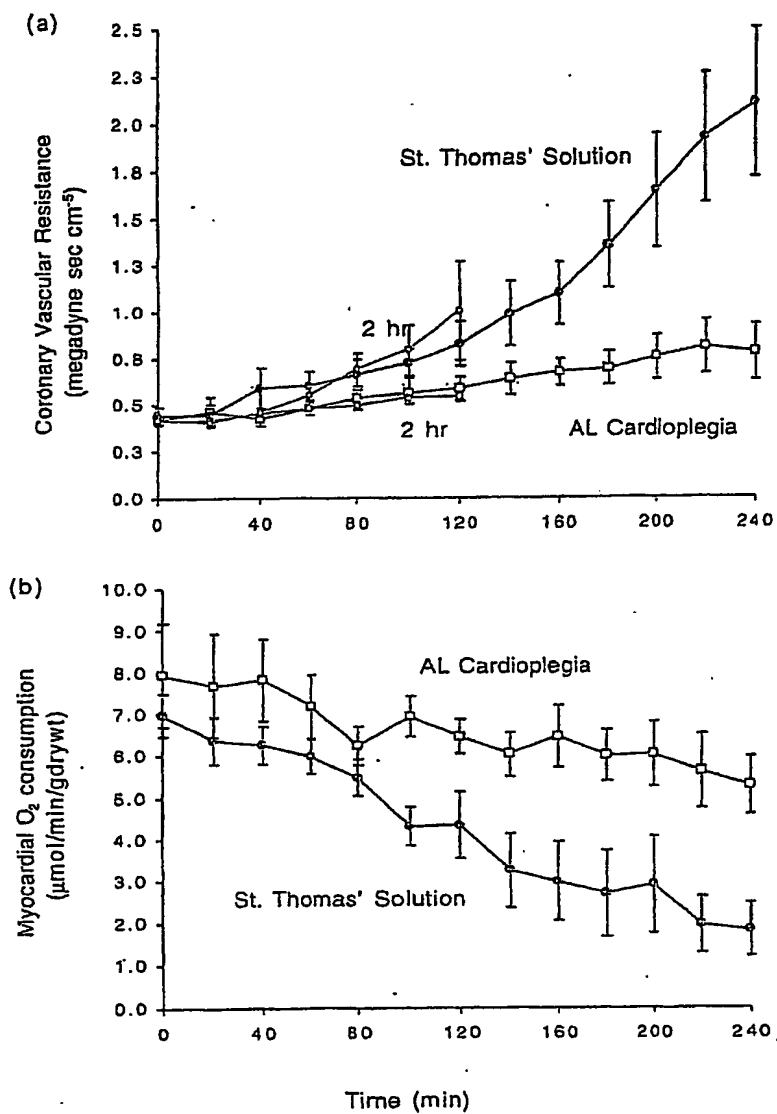


Figure 6

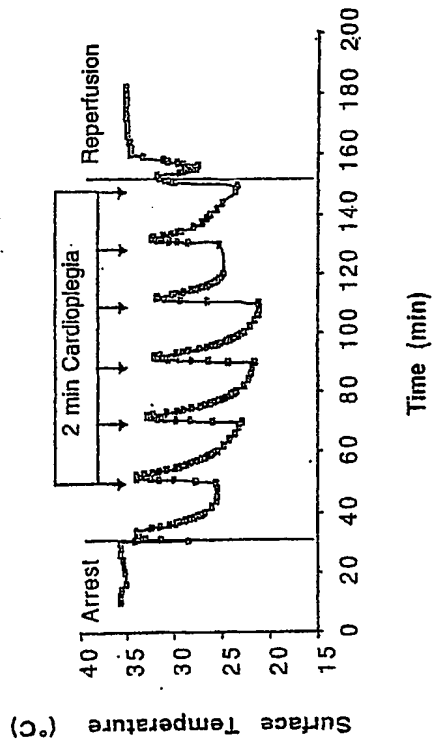


Table 1 Estimates of the membrane potential (in millivolts) in the isolated rat heart before and during arrest by adenosine and lidocaine (AL) cardioplegia¹ (5.9 mM K⁺), hyperkalemic St. Thomas Hospital solution No 2 (16 mM K⁺) or 16 mM KCl at 37°C. Values are mean \pm SEM.

Treatment	No of hearts	Membrane potential (This study)	Published Values	References
Normal ⁵ Pre-Arrest Controls	6	-83 \pm 2 mV ³	-84 \pm 2 mV ²	Masuda, T, Dobson, GP and , RL (1990) J. Biol. Chem. 265 (33) 20321-34
St. Thomas Solution #2	6	-48 \pm 3 mV ³	-84 ⁴ \pm 1 mV ^{2,4}	Kleber AG (1983) Circ Res. 52 (4) 442-50
16 mM KCl (8°C)	7		-50 mV ²	Chambers DJ (1999) Curr Opin Cardiol 14 (6) 495-500
16 mM KCl	6		-49.5 \pm 1 mV ^{2,4}	Snabaitis, AK, Shattock, MJ, and Chambers, DJ (1997) Circulation 96 (9) 3148-56
AL Cardioplegia	6	-83 \pm 1 mV ³		Kleber AG (1983) Circ Res. 52 (4) 442-50

¹ Adenosine (200 μ M) and lidocaine (500 μ M) was in 10 mM glucose-containing Krebs-Henseleit solution pH 7.4

² Measured using 3M KCL microelectrodes

³ Membrane potential was calculated from the Nernstian distribution of K⁺ ion between intra- and extra-cellular compartments of left ventricle as described in Masuda, Dobson and Veech (1990) The Donnan Near-Equilibrium system of heart. J. Biol. Chem 265 (33) 20321-34

⁴ Isolated perfused guinea pig heart.

⁵ Healthy (non-injured) pre-arrest perfused isolated rat hearts in the working mode

Table 2 Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 2 hour arrest with adenosine-lidocaine (AL) cardioplegia or modified St. Thomas' solution (St.T).

2 hour Arrest Protocol	Treatment	n	Heart Rate (bpm)	Aortic Flow (ml/min)	Coronary Flow (ml/min)	Rate Pressure Product (mmHg/min)	O ₂ Consumption (μmol/min/g dry weight)®
5 min Pre-Arrest	AL	7	259 ± 20	33.2 ± 2.7	17.1 ± 1.8	30998 ± 2046	45.3 ± 4.30
	St.T	8	259 ± 13	34.5 ± 2.1	18.0 ± 1.3	31329 ± 1720	46.1 ± 2.60
15 min Recovery	AL	7	215 ± 24	17.0 ± 3.6	15.3 ± 1.4	24934 ± 2506	53.6 ± 7.2
	St.T	8	108 ± 32*	5.9 ± 3.8	7.3 ± 2.9*	9514 ± 3737*	16.4 ± 6.6
30 min Recovery	AL	7	248 ± 22	25.5 ± 2.3	15.4 ± 1.6	28722 ± 2149	51.6 ± 5.6
	St.T	8	148 ± 47*	9.4 ± 7.0*	8.93 ± 4.6	12498 ± 6863*	18.9 ± 7.5
60 min Recovery	AL	7	245 ± 26	24.6 ± 2.7	13.8 ± 1.7	27958 ± 2457	49.8 ± 6.5
	St.T	8	147 ± 45*	7.7 ± 5.9*	8.35 ± 4.4	11808 ± 6533*	18.8 ± 7.8

* denotes significance between treatment groups p<0.05 ** denotes significance between treatment groups p<0.001

®To convert from μmol/min/g dry weight to wet weight divide by 7.46 for both pre-arrest groups, and by 9.26 (AL hearts) and 7.41 (St. Thomas' hearts) in recovery (see text for details).

Table 3 Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 4 hour arrest with adenosine-lidocaine (AL) cardioplegia or modified St. Thomas' solution (St.T).

4 hour Arrest Protocol	Treatment	n	Heart Rate (bpm)	Aortic Flow (ml/min)	Coronary Flow (ml/min)	Rate Pressure Product (mmHg/min)	O ₂ Consumption (μ mol/min/g dry weight) [*]
5 min Pre-Arrest	AL	9	275 \pm 13	36.5 \pm 1.7	16.28 \pm 1.0	32338 \pm 1084	50.3 \pm 3.4
	St.T	7	259 \pm 13	41.2 \pm 4.2	16.03 \pm 1.3	31508 \pm 1672	57.2 \pm 1.8
15 min Recovery	AL	9	229 \pm 16	19.8 \pm 3.6	13.9 \pm 1.5	25327 \pm 1555	55.0 \pm 6.4
	St.T	7	67 \pm 28**	2.7*	2.3**	3815 \pm 3040**	5.7 \pm 5.1**
30 min Recovery	AL	9	239 \pm 19	24.6 \pm 2.9	11.5 \pm 1.0	26684 \pm 1669	45.7 \pm 4.1
	St.T	7	79 \pm 26**	2.4**	2.9*	4137 \pm 3170 **	6.1 \pm 5.5**
60 min Recovery	AL	9	249 \pm 17	25.6 \pm 3.3	11.4 \pm 1.3	27569 \pm 1577	44.6 \pm 4.8
	St.T	7	83 \pm 30**	2.1**	2.6*	4359 \pm 3527**	7.1 \pm 6.5**

* denotes significance between treatment groups $p < 0.05$ ** denotes significance between treatment groups $p < 0.001$
 # Only 1 of 7 St Thomas' hearts had measurable aortic and coronary flows and only the mean values are presented.
 * To convert from μ mol/min/g dry weight to wet weight divide by 7.46 for both pre-arrest groups, and by 9.26 (AL hearts) and 7.41 (St. Thomas' hearts) in recovery (see text for details).

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☒ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.